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Evidence of a Cellular Immune Response Against Sialyl-Tn in Breast and Ovarian Cancer Patients After High-Dose Chemotherapy, Stem Cell Rescue, and Immunization with Theratope STn-KLH Cancer Vaccine

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Summary: Seven ovarian and 33 breast high-risk stage II/III and stage IV cancer patients received high-dose chemotherapy followed by stem cell rescue. Thirty to 151 days after stem cell transplantation, the patients received their first immunotherapy treatment with Theratope STn-KLH cancer vaccine. Most patients developed increasing IgG anti-STn titers to a sustained peak after the fourth or fifth immunizations. Only one patient had elevated CA27.29 (MUC1 mucin) serum levels at trial entry. Five of the seven patients with preimmunotherapy elevated serum CA125 levels demonstrated decreasing CA125 levels during immunotherapy, consistent with an antitumor response. Evidence of STn antigen-specific T-cell proliferation was obtained from 17 of the 27 evaluable patients who received at least three immunotherapy treatments. Eleven of the 26 patients tested had evidence of an anti-STn TH_1 antigen-specific T-cell response as determined by interferon-γ, but not interleukin (IL)-4, production. After immunization, lytic activity of peripheral blood lymphocytes (PBLs) tested against a lymphokine activated killer (LAK)-sensitive cell line, a natural killer (NK)-sensitive cell line, and an STn-expressing cancer cell line (OVCAR) increased significantly. In vitro IL-2 treatment of the PBLs after vaccination greatly enhanced killing of the STn^+ cancer cell line. Evidence of the development of OVCAR specific killing activity, over and above that seen due to LAK or NK killing, is presented. These studies provide the strongest evidence in humans of the development of an antitumor T-cell response after immunization with a cancer-associated carbohydrate antigen. **Key Words:** Carbohydrate antigen—Cellular immune response—Immunotherapy—Sialyl-Tn—Stem cell rescue.

Active specific immunotherapy (ASI) holds a great deal of promise as a novel, nontoxic treatment of human cancers. Numerous animal and human studies have demonstrated that various cancer vaccines can stimulate antibody and cell-mediated immune responses against tumor-associated antigens (1–9). Nevertheless, very few studies with cancer vaccines have demonstrated convincing clinical responses. One of the reasons for this might be due to the production by tumors of immunosuppressive factors that can limit both the magnitude and the efficacy of the immune response generated after immunization with a cancer vaccine.

Sialyl-Tn $(STn)^+$ MUC1 mucins are potentially immunosuppressive molecules produced and shed by tumors. STn is a disaccharide antigen that is expressed on the

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majority of human cancers (10) and is associated with the MUC1 mucin core peptide (11). Several clinical studies indicate that patients whose tumors express or secrete high levels of STn have a particularly poor prognosis (12–16), and other studies have shown that mucins shed by tumors are immunosuppressive (17–19).

Based on the demonstration that STn is associated with a poor prognosis and immunosuppression, the STn epitope is an appropriate target for ASI. To date, >325 patients with metastatic adenocarcinoma have been treated in clinical trials (6–9,18,20–22) with the Theratope STn-KLH cancer vaccine in the non–stem cell rescue setting. In these studies, the cancer vaccine was formulated using a synthetic sialyl-Tn epitope with a linker arm conjugated to keyhole limpet hemocyanin (KLH). The STn-KLH conjugate was emulsified in DETOX-B SE adjuvant and injected subcutaneously on weeks 0, 2, 5, and 9, followed by monthly and then every-3-month boosters in stable or responding patients. Breast cancer patients pretreated with low-dose intravenous cyclophosphamide followed by immunotherapy generated higher antibody titers against the STn epitope and had longer survival after immunization with Theratope STn-KLH cancer vaccine compared with patients who received no cyclophosphamide or low-dose oral cyclophosphamide before immunotherapy (8,9,22). Breast, ovarian, and colorectal cancer patients who produced the highest anti-STn IgG titers after immunotherapy with Theratope STn-KLH cancer vaccine survived longer than patients who produced lower titers after immunization (8). This correlation of the IgG antibody response to survival was specific for mucin-expressed STn, consistent with the hypothesis that antibody against STn generated by the cancer vaccine reacts with mucin-associated STn expressed on tumor cells or on shed mucins and provides a therapeutic benefit for the patient. Possible cellular immune responses to STn were not followed in these studies. Nevertheless, the efficacy of similar cancer vaccines in animal models correlated with the magnitude of the delayed-type hypersensitivity reaction to the Tn or Thomsen-Friedenreich (TF) carbohydrate antigen in the vaccine and represented on tumor-associated mucins present on tumors (23,24). It has recently been confirmed that T cells can react specifically against carbohydrate antigens (25–30). In addition, experiments demonstrating a high degree of carbohydrate specificity in T-cell recognition of the tumor-associated Tn antigen (31) have been reported (30).

Theoretically, immunotherapy is most effective when used in patients with a relatively low tumor burden. In the present study, high-risk stage II and III and metastatic stage IV breast cancer and stage III and IV ovarian cancer patients who underwent high-dose chemotherapy followed by stem cell rescue were treated with Theratope STn-KLH cancer vaccine. This group of patients were thought to be potentially good candidates for an immunotherapy trial as the majority of these patients would be expected to undergo a partial or complete response to high-dose chemotherapy. Consequently, these patients would have relatively low tumor burdens and, as a result, would not be expected to produce high levels of immunosuppressive MUC1 mucin (18,19), which can cause T-cell anergy (19). On the other hand, it is well documented that such patients are immunosuppressed for antibody and cellular immune responses (32). Therefore, the present study was designed to study the immune response of these patients, both cellular and humoral, after high dose chemotherapy followed by stem cell rescue and multiple immunizations with Theratope STn-KLH cancer vaccine.

MATERIALS AND METHODS

Study Design and Patient Enrollment

Informed consent was obtained by using consent forms approved by the Institution Review Board of Fred Hutchinson Cancer Research Center (FHCRC). Eligible patients for this study included those who underwent high-dose chemotherapy and autologous (39 patients) or syngeneic (1 patient) peripheral blood stem cell (PBSC) rescue for breast or ovarian cancer. Three patients (patients 20, 33, and 39) received an interleukin (IL)-2-incubated PBSC product, and two (patients 33 and 39) of the three patients received posttransplant IL-2 infusion on days 0–5. Posttransplant hematopoietic growth factors [i.e., granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF, etc.] are not routinely given to these patients, and none of the patients was receiving growth factors at the time of immunization. Breast cancer patients eligible for high-dose chemotherapy and stem cell rescue included patients with stage IV breast cancer, patients with inflammatory (IIIb) breast cancer or high-risk stage IIa, IIb, or III patients with ≥ 4 axillary lymph nodes involved with tumor. Ovarian cancer patients included those transplanted for stage III or IV disease. Patients had to have a performance status of $\geq 70\%$ (Karnovsky score), an absolute neutrophil count (ANC) $>1.5 \times 10^9$/L, and platelets (self-supporting) $>20 \times 10^9$/L. If a patient relapsed while on study, she could continue on study if she were not receiving other nonhormonal therapy for her disease. All patients received Theratope STn-KLH cancer vaccine with DETOX-B SE (Ribi Immunochem Research Inc., Hamilton, MT,

U.S.A.) adjuvant as described previously (8,9,18). The emulsified vaccine in a total volume of 0.6 ml was injected subcutaneously, 0.3 ml injected in each of two sites in the upper arms, thighs, or abdomen. Patients were scheduled to receive a total of five immunizations during the 1st year posttransplant. The first immunization occurred from day 30 to day 151 posttransplant. The second immunization was given 2 weeks after the first immunization and subsequent immunizations were spaced at approximately every 2–3-month intervals (Table 1).

TABLE 1. *Immunization schedule*

		Immunization no.					
Pt. no.	Diagnosis	1	2	3	4	5	Status
1	Br	92[a]	106	166	267		R[b]
2	Ov	49	77	133	189	245	C[c]
3	Br	67	81	151	218	288	C
4	Ov	37	51	131	215		W[d]
5	Br	93	111	188	247	310	C
6	Br	119	133	207	364	567	C
7	Br	30	48	124	216	307	C
8	Ov	89					W
9	Br	134	148	214	305	368	C
10	Br	149					W
11	Br	42	55	108	189	245	C
12	Br	91	105	161	238	301	C
13	Br	125	142	222	306	362	C
14	Br	148	162	222	284	361	C
15	Ov	144	158	232	337	404	C
16	Br	39	53	151			W
17	Br	150	168	253	364	454	C
18	Br	143	158	243			R
19	Br	59	73	137	207	270	C
20	Br	140	154	213	283	360	C
21	Br	148	162	236	312	407	C
22	Br	139	154	210	272	342	C
23	Br	138	151	223	347	445	O[e]
24	Br	66	80	150	220		R
25	Br	147	161	262	346	416	C
26	Br	138	156	226	296	356	C
27	Br	144	162	224	294	382	C
28	Br	87	101				R
29	Ov	150	167	237	300	377	C
30	Br	146	160				R
31	Ov	95	109	179			R
32	Br	32	46	133	217		O
33	Br	37	55	107	184	268	C
34	Br	143	157				R
35	Br	151	164	238	319		O
36	Br	150	171	231	321		O
37	Br	139	154				R
38	Br	101	116				R
39	Ov	49	70				R
40	Br	130	145	213			O

Br, breast cancer; Ov, ovarian cancer.
[a] Days after stem cell transplant.
[b] Relapsed and withdrew.
[c] Completed trial, being monitored for survival.
[d] Withdrew from trial.
[e] Still on trial.

Immunology Studies

During each visit for immunizations, blood was drawn for serum antibody titrations and cellular assays just before receiving immunotherapy. Blood was also collected 2 weeks after immunotherapy in a subset of patients.

Assays for Serum Tumor Marker Levels

The Truquant BR radioimmunoassay (RIA) (33,34) was used to measure CA27.29 antigen (MUC1) levels in the serum and Truquant OV RIA was used to measure CA125 antigen levels (35). Greater than 37 U/ml is considered to be an elevated serum level for both assays.

Assays for Antibody

Clotted blood was clarified and sera collected, aliquoted, and stored at −80°C. Thawed aliquoted samples were evaluated for IgM and IgG to OSM, STn-HSA (human serum albumin), and KLH, which were used as target epitopes in solid-phase enzyme-linked immunosorbent assays (ELISA) before and after treatment with Theratope STn-KLH cancer vaccine as previously described (8,9).

Cellular Assays

Peripheral blood lymphocytes (PBLs) were isolated from a freshly drawn heparinized tube of blood. Lymphocytes were isolated after density gradient separation on Ficoll-Hypaque and washed two times in Hanks' balanced salt solution, then two times in HBSS with 5% fetal bovine serum and resuspended in 1.0–2.0 ml of AIM-V serum-free media. All cellular assays were conducted on freshly isolated PBL.

Proliferation Assays

The cell concentration was adjusted to 4×10^6 cells/ml in AIM-V media for STn antigen-induced T-cell proliferation and cytokine production. One hundred microliters of the cell suspension was added per well (performed in six replicates for each patient sample) of a round-bottom 96-well microtiter tissue culture plate. Forty micrograms per milliliter of KLH, HSA, or STn-HSA was added to the cells in 100-μl AIM-V serum-free media followed by incubation of the culture plate at 37°C in a humidified CO_2 incubator for 72 h. Proliferation to STn-HSA versus controls (HSA and KLH) was measured side by side in the same experiments. One hundred fifty microliters of the supernatant from each well was placed into sterile 2-ml cryovials, and the supernatants were

stored at −20°C until assayed for IL-4 and interferon (IFN)-γ as previously described (36). Twenty microliters of [^{3}H]thymidine at a concentration of 1 μCi/20 μl diluted in AIM-V media was then added to each well and incubated for 18 h at 37°C in a humidified CO_2 incubator. The contents of the wells were then harvested onto filter mats using a cell harvester (PHD) and counted in a scintillation counter (Beckman LS 6000).

Cytotoxic Assays

Daudi, K562, and OVCAR cells were obtained from the American Type Culture Collection (Bethesda, MD, U.S.A.). OVCAR is an ovarian cancer cell line that expresses STn on the cell surface as determined by fluorescence activated cell sorter (FACS) analysis with the STn-specific antibody B195 (11). The level of OVCAR STn expression was significantly enhanced after three passages of the OVCAR cell line as a solid tumor in nude mice. FACS staining revealed an increase in STn expression from 39% positive to 79% positive cells and an increase in staining intensity of 72% (data not shown). The target cells were grown in RPMI 1640 media to yield 4–8 × 10^5 cells/ml for Daudi or K562 or 4–7 × 10^6 cells/ml for OVCAR-3 cells adherent in a 25-cm^2 tissue culture flask. The nonadherent Daudi or K562 cells were placed in a disposable 15-ml centrifuge tube. For OVCAR-3 cells, the supernatant was removed and replaced with 4 ml of a fresh trypsin-EDTA solution for ~1 min; then the trypsin-EDTA solution was removed and replaced by fresh AIM-V media, pipetted up and down vigorously to dispense clumps of cells, and then transferred to 15-ml centrifuge tubes. Target cells were washed two times in HBSS with 10% fetal calf serum (FCS) + 1% bovine serum albumin and resuspended in AIM-V media. The target cells were then labeled with 200 μCi of ^{51}Cr for 90 min in a humidified CO_2 incubator at 37°C. The cells were gently resuspended every 20 min during the incubation period. The labeled cells were washed four times in 10 ml of cold (4°C) media with 10% FCS. After the last spin, the ^{51}Cr-labeled cells were resuspended in 1 ml of AIM-V media for each 10^6 cells labeled, and a viability cell count was done followed by resuspending the cells at a concentration of 10^5 cells/ml in AIM-V media. The effector PBLs were either not stimulated with IL-2 or stimulated at a concentration of 10^6 cells/ml in a 24-well plate with 100 U/ml of human IL-2 (Genzyme, Inc.) for 48–72 h at 37°C in a humidified CO_2 incubator after which the cells were harvested, washed, resuspended in 1–2 ml of AIM-V media, and counted. The effector cell concentration was adjusted to 10^6 cells/ml for use at a 10:1 effector:target cell ratio (10^4 target cells). Cytotoxicity assays were performed in triplicate for each patient sample. Spontaneous-release control wells contained 100 μl of AIM-V media and 100 μl of labeled target cells (10^4) and maximum ^{51}Cr release wells contained 90 μl AIM-V, 10 μl of 4% NP-40, and 100 μl of labeled target cells (10^4). The assay plate was centrifuged at 100 *g* for 3 min and incubated at 37°C in a humidified CO_2 incubator for 4 h, after which 100 μl of cell supernatant was removed for counting in a Packard Cobra gamma counter.

Patients

Table 1 summarizes the patients' status in terms of their diagnosis, the dates they received their immunizations after stem cell transplantation, as well as their clinical status through August 1997. There were 7 ovarian cancer patients and 33 breast cancer patients. They received their first immunization anywhere from day 30 to day 151 after stem cell transplantation. Twenty-two patients have completed all five ASI treatments and are currently being monitored for survival. Four patients are still on trial and 4 patients have withdrawn from the trial before they could receive their complete series of vaccinations. Ten patients have relapsed before completing their therapy and withdrew from the trial to receive chemotherapy.

Statistical Analysis

Student's *t* test to compare groups, paired *t* tests to compare paired data, and correlation analysis were done using a STATVIEW statistical program (37).

RESULTS

Anti-OSM Antibody Titers in Patients Who Received at Least Three Immunizations with Theratope STn-KLH Cancer Vaccine

Solid-phase ovine submaxillary mucin (OSM) was used to estimate the antibody titers against mucin-associated STn (6–9). The values listed in Table 2 represent the peak antibody titers in 30 evaluable patients who were tested after having received at least three immunizations. The median peak anti-OSM IgG $\log_2$ titer of all these patients was 7.322, which corresponds to a geometric median titer of 1:160. Sixteen of the 30 evaluable patients had a peak anti-OSM IgG titer ≥1:160. Figure 1 illustrates the dynamics of the anti-OSM IgG and IgM antibody responses in representative patients who achieved peak IgG titers of 1:160 or greater. In patients 5, 14, 21, and 29 (patients 5 and 14 are illus-

TABLE 2. *Peak anti-ovine submaxillary mucin (OSM) antibody titers*[a]

Pt. no.	No. of immunizations	IgM	IgG
1	4	10 (3)[b]	5 (3)
2	5	20 (4)	40 (4)
3	5	5 (5)	40 (5)
4	4	160 (4)	1,280 (4)
5	5	320 (5)	1,280 (5)
6	5	80 (5)	320 (5)
7	5	40 (5)	160 (4)
9	5	20 (2)	80 (5)
11	5	80 (5)	40 (5)
12	5	40 (4)	5 (4)
13	5	640 (4)	1,280 (4)
14	5	160 (3)	160 (4)
15	5	40 (3)	1,280 (4)
16	3	5 (3)	10 (3)
17	5	10 (5)	0 (5)
19	5	40 (3)	640 (5)
20	5	320 (4)	640 (5)
21	5	160 (4)	160 (4)
22	5	40 (2)	160 (4)
23	5	320 (2)	40 (4)
24	4	10 (4)	20 (4)
25	5	80 (4)	320 (4)
26	5	40 (5)	320 (5)
27	5	160 (4)	160 (5)
29	5	80 (2)	320 (4)
31	3	320 (3)	10 (3)
32	4	10 (3)	20 (3)
33	4	40 (4)	80 (4)
35	4	20 (3)	160 (1)
36	4	320 (4)	80 (4)

[a] Peak anti-OSM antibody titers of patients who were tested after at least three immunizations.

[b] Reciprocal of dilution of serum giving peak antibody titers (after [no.] of immunizations).

trated in Fig. 1), there was a classic increase in IgM followed by an increase in IgG titers. In patients 13, 14, 15, 19, and 21 (patients 13, 14, and 15 are illustrated in Fig. 1), there were increasing sustained titers after each immunization to an apparent plateau. Some of the patients (patients 4, 6, 7, 20, 22, 25, 26, and 27) showed an increase in anti-OSM IgG titer only after 3, 4, or 5 immunizations (patients 7 and 20 are illustrated in Fig. 1). Patient 35 was unusual because she had a preimmunotherapy anti-OSM IgG titer of 1:80, which only increased to 1:160 after the first immunization and did not change after a further three immunizations (data not shown). All other patients had a pre-ASI anti-OSM IgG titer $\leq$1:10 (28 of 40 patients had a pre-ASI titer of 0).

The peak IgG titers measured using the STn-HSA solid phase (anti-STn IgG) ranged from 0 to 1:10,000 (geometric median titer = 1:640), and the peak anti-KLH IgG titers ranged from 1:40 to < 1:10,000 (geometric median titer = 1:10,000) (data not shown). There was a significant positive correlation between the anti-OSM IgG titers and the anti-STn IgG titers (R = 0.650, $p < 0.0001$). There was no significant correlation between the anti-OSM IgG titers and the anti-KLH IgG titers (R = 0.304, p = 0.102).

Tumor Marker Serum Levels Before and After Immunization

Only one of the 40 patients (patient 38) had elevated (>200 U/ml) serum CA27.29 (MUC1) before immunotherapy. Five (patients 2, 3, 24, 37, and 38) of the 40 patients demonstrated elevated levels of serum CA27.29 during immunotherapy. Seven of the 40 patients had elevated serum CA125 levels before immunotherapy. Of the seven patients with preimmunotherapy elevated serum CA125 levels, five patients (patients 1, 16, 20, 32, and 36), demonstrated decreasing CA125 levels during immunotherapy (Fig. 2), and the remaining two patients (patients 3 and 37) showed further increases in CA125 during immunotherapy. An additional four patients (patients 2, 17, 30, and 31) demonstrated elevated levels of serum CA125 during immunotherapy.

T-Cell Proliferative and Cytokine Responses After Immunotherapy

Table 3 summarizes the T-cell proliferation data from 27 patients who were tested after at least three immunizations. The data represent the actual counts per minute (CPM), from the T-cell cultures stimulated with HSA or STn-HSA and the stimulation indices for the specific T-cell proliferation to STn-HSA, STn-KLH, and KLH. In each case, the stimulation index was calculated by dividing the counts per minute achieved with HSA control cultures into counts per minute achieved with either STn-HSA, STn-KLH, or KLH cultures. For comparative purposes, nine normal donors were tested for their stimulation index to STn-HSA. The stimulation index (SI) of the nine normal donors was 1.61 $\pm$ 0.37 (mean $\pm$ standard deviation). The anti-STn SI of the 38 cancer patients tested just before receiving the first ASI was 1.103 $\pm$ 0.37 (data not shown), and the peak SI of the 27 patients who had received at least three ASI treatments was 4.406 $\pm$ 5.593 (paired t test, t = 3.026, p = 0.0058, comparison of the pre-ASI SI and the peak SI for the patients who received at least three immunizations). The mean + 2 SD of the normals, which equals a stimulation index of 2.35, was used to define the upper limit of normals. It can be seen from Table 3 that 17 of 27 patients who received at least three immunizations had a peak stimulation index

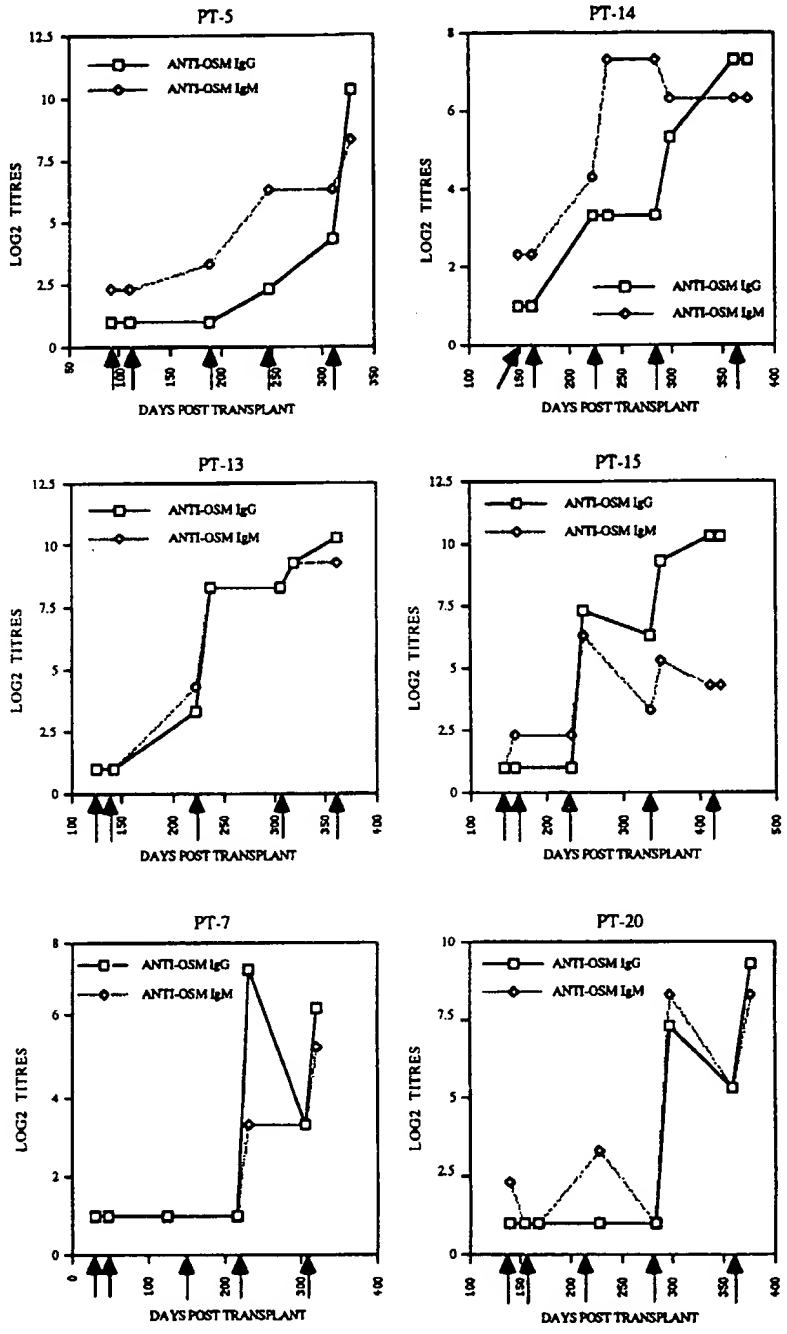

FIG. 1. Dynamics of the anti-ovine submaxillary mucin (OSM) IgG and IgM response following immunization with Theratope STn-KLH cancer vaccine. The $\log_2$ titers as a function of time after each immunization are shown. The arrows indicate the time after each immunization for each of the patients illustrated. Patients 5 and 14 are illustrative of patients who showed an IgM response followed by an IgG response. Patients 13 and 15 are illustrative of patients showing a gradual increase in antibody IgG anti-OSM titer to an apparent plateau. Patients 7 and 20 are illustrative of patients who did not show a significant IgG and anti-OSM response until after the fourth immunization.

greater than the upper limit of normals, 2.35. The dynamics of the response of the 12 patients who had peak anti-STn SI of 3 or greater is illustrated in Fig. 3. It can be seen that in all 12 patients, the peak anti-STn SI in PBL occurred after the third ASI treatment. Most of the patients also developed strong T-cell proliferation in response to KLH and STn-KLH (Table 3).

Antigen-Specific Cytokine Responses

Twenty-six of the 40 patients in the study were tested for antigen-specific cytokine responses after immunotherapy. IFN-γ was followed as a TH_1-type cytokine and IL-4 as a TH_2-type cytokine. No STn antigen-specific IL-4 responses were detected in any of the 26 patients

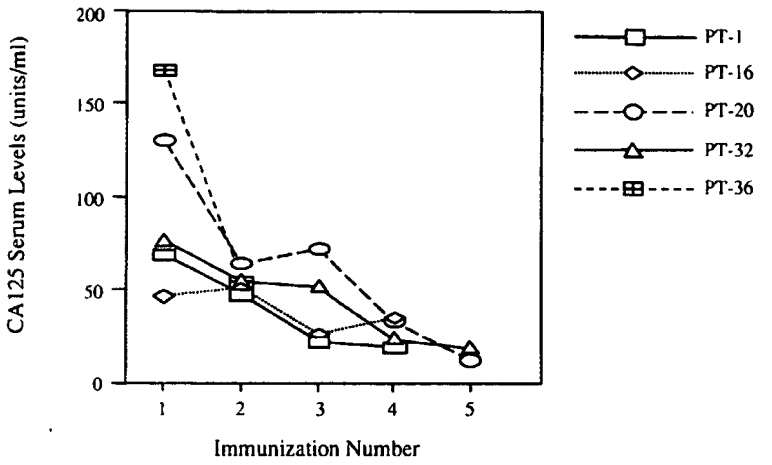

FIG. 2. CA125 serum levels as a function of when the serum sample was drawn for CA125 testing. The immunotherapy number is indicated on the *x* axis. The blood sample for CA125 analysis was taken just before each immunotherapy treatment. Illustrated are five patients who had elevated (>37 U/ml) CA125 just before immunotherapy treatment 1 and who showed a decrease in CA125 levels while receiving immunotherapy with Theratope STn-KLH cancer vaccine.

(data not shown). The amount of IFN-γ produced by experimental (STn-HSA or STn-KLH) and control (HSA or KLH) wells was compared. A positive biologic response was defined as experimental wells that produced at least two times the amount of cytokine in the control well. Eleven of the 26 patients tested had evidence of anti-STn antigen-specific T-cell stimulation as determined by IFN-γ production (Table 4). The T cells from five patients secreted at least two times more IFN-γ in response to STn-HSA compared with HSA. Eight patients produced at least two times more IFN-γ in the presence of STn-KLH than in the presence of KLH. None of the patients had responses where the amount of cytokine produced in the HSA or KLH cultures was greater than the amount of cytokines produced in the parallel STn-HSA or STn-KLH cultures, respectively. There was no correlation between the peak stimulation indices and IFN-γ secretion (data not shown).

TABLE 3. *Summary of peak T-cell proliferation data*[a]

	CPM		Stimulation index[b]		
Pt. no.	HSA	STn-HSA	STn-HSA	STn-KLH	KLH
1	1,072	1,123	1.05	10.3	8.40
2	814	1,424	1.70	9.8	10.3
3	331	547	1.70	3.13	2.82
4	2,117	6,616	3.10	21.4	22.9
5	810	1,784	2.20	61.3	61.1
6	2,919	7,551	2.60	4.3	3.6
7	839	4,040	4.80	6.95	7.78
9	3,344	4,167	1.20	6.6	6.1
11	2,203	2,597	1.20	3.50	3.90
12	1,654	4,211	2.55	2.90	4.70
13	251	6,316	25.2	20.5	20.0
14	1,787	1,546	.96	12.5	14.5
15	3,093	3,740	1.20	9.3	9.35
17	1,430	1,893	1.30	6.5	5.2
19	143	390	2.70	11.5	10.6
20	188	2,464	13.1	49.7	49.6
21	1,018	3,073	3.02	13.6	11.8
22	2,964	10,831	3.65	6.80	6.70
23	2,317	6,562	2.80	2.60	4.40
24	189	3,495	18.5	16.6	3.90
25	1,457	3,648	2.50	8.70	6.90
26	139	461	3.30	3.30	3.30
27	263	1,146	4.37	6.2	5.70
29	2,912	10,881	3.70	9.8	9.2
32	1,322	2,544	1.90	2.70	1.80
33	1,214	4,039	3.30	4.50	3.70
36	447	1,962	4.40	7.50	6.70

[a] For patients tested after at least three immunizations.
[b] Up to and including the fifth active specific immunotherapy treatment. Stimulating index is calculated relative to HSA counts per minute.

Cellular Cytotoxic Activity of PBLs from Immunotherapy Patients

PBLs from patients and normal donors were preincubated in the presence (+IL-2) or absence (−IL-2) of IL-2, and then tested in parallel for lytic activity against K562, Daudi, and OVCAR cells. In Table 5, it can be seen that NK activity (K562-IL-2) and anti-OVCAR (−IL-2) lytic activity of the patients before immunization was significantly lower than that of normal donors, whereas lymphokine activated killer cell (LAK) (Daudi + IL-2) lytic activity was not significantly different. For all other tests (K562 + IL-2, Daudi − IL-2, and OVCAR + IL-2), patients did not differ significantly from the normal donors before immunization (Table 5). After immunization, NK and LAK lytic activity increased significantly (Table 5). OVCAR (−IL-2) lytic activity also increased after immunotherapy, although there was no significant increase in killing of the STn^- Daudi cells (−IL-2). This suggests that there was an increase in specific killing of the STn^+ OVCAR cancer cell line after immunotherapy. This is further illustrated in Table 6. Six of the patients developed especially potent killing of OVCAR cells after im-

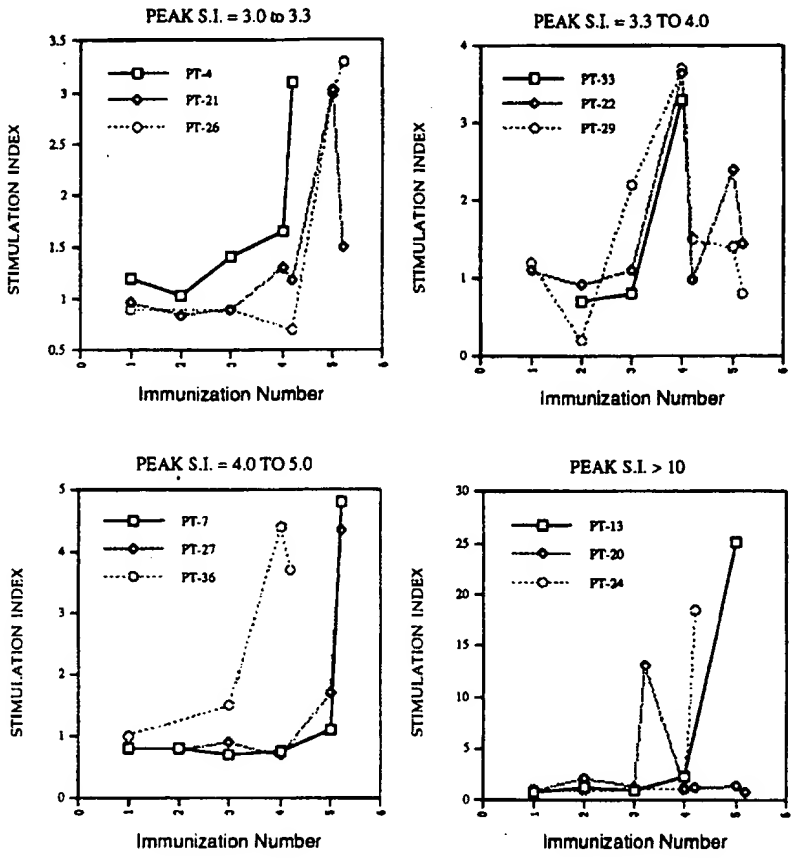

FIG. 3. Dynamics of the anti-STn specific proliferative response after immunotherapy with Theratope STn-KLH cancer vaccine. Four categories of patients are shown in the figure for ease of illustration: three patients who had a peak stimulation index of 3–3.3, three patients who had a peak stimulation index of 3.3–4, three patients who had a peak stimulation index of 4–5, and three patients who had a peak stimulation of >10. The active specific immunotherapy number at which the blood sample was drawn for the T-cell proliferation analysis is shown in the *x* axis and, on the *y* axis, the stimulation index as measured by the T-cell proliferation with STn-HSA in the culture over the T-cell proliferation induced with just HSA in the culture.

munotherapy (22.6–70.1% specific lysis) that was far greater than the peak lysis of Daudi cells by the same patient. There was also an increase in "IL-2 assisted" killing of OVCAR target cells after immunotherapy that was greater than the increase in "IL-2 assisted" killing of Daudi cells (LAK killing) (Table 7). In Table 7, we examine the magnitude of the STn^+ cancer cell line "IL-2 assisted" OVCAR specific killing by comparing the peak percent lysis of OVCAR cells minus the peak percent IL-2 assisted lysis of Daudi cells (LAK lysis). Using this method, only one patient (patient 31) had a >5% increase in LAK lysis compared with OVCAR (+ IL-2) lysis after immunotherapy. In contrast, 14 of the 29 evaluable patients had >10% OVCAR lysis when the peak LAK lysis is subtracted from the peak "IL-2 assisted" OVCAR lysis for each patient. Some representative examples of the dynamics of the "IL-2 assisted" anti-OVCAR response are shown in Fig. 4. Peak responses noted after one, two, or three immunizations are illustrated. In 12 of 18 evaluable patients who had at least three tests after immunotherapy, there was an increase and then decrease in "IL-2 assisted" OVCAR lytic activity in PBLs.

TABLE 4. *Antigen specific interferon (IFN)-γ responses*

Pt. no.	After immunization no.	HSA	STn (HSA)[a]	KLH	STn (KLH)[b]
1	3	—	—	0	35
5	3	—	—	74	148
12	4	—	—	0	36
13	4	—	—	184	497
17	3	—	—	0	36
20	2, 5	0	30	0	65
21	5	—	—	29	81
22	2	0	32	—	—
24	1	0	35	—	—
27	4	75	733	—	—
29	2	0	125	0	39

[a] pg/ml IFN-γ generated in STn-HSA-stimulated cultures.
[b] pg/ml IFN-γ generated in STn-KLH-stimulated cultures.

DISCUSSION

In the present study, high-risk stage II/III and stage IV breast cancer and stage III/IV ovarian cancer patients

TABLE 5. *K562, Daudi, and OVCAR lysis in normals versus patients*[a]

Target cell	PBL preincubated[b]	Normals	1st test patients[c]	Peak post ASI
K562	−IL-2 (NK)	22.6 ± 3.1 (9)[d]	11.0 ± 2.5 (36)[d,e]	15.2 ± 2.4 (36)[e]
	+IL-2	55.2 ± 5.4 (9)	39.6 ± 4.8 (22)[f]	55.8 ± 3.3 (33)[f]
Daudi	−IL-2	4.1 ± 0.9 (9)	5.9 ± 2.5 (27)	6.3 ± 1.6 (30)
	+IL-2 (LAK)	27.7 ± 3.6 (9)	26.4 ± 5.0 (22)[g]	35.1 ± 3.7 (30)[g,l]
OVCAR	−IL-2	7.1 ± 2.7 (10)[h]	3.1 ± 0.5 (29)[h,i]	13.3 ± 3.4 (26)[i]
	+IL-2	30.2 ± 5.3 (12)[j]	21.0 ± 3.3 (25)[k]	47.2 ± 4.3 (32)[j,k,l]

PBL, peripheral blood lymphocytes; ASI, active specific immunotherapy; NK, natural killer cells; IL-2, interleukin-2; LAK, lymphokine activated killer cells.

[a] Mean % ^{51}Cr release ± SEM (n).

[b] PBL preincubated with or without IL-2 before cytotoxic killing assay.

[c] First test was either just before the first or second immunotherapy treatment, see Material & Methods.

[d] Significantly different, p = 0.034, unpaired *t* test.

[e] Significantly different, p = 0.001, paired *t* test.

[f] Significantly different, p = 0.003, paired *t* test.

[g] Significantly different, p = 0.003, paired *t* test, 19 common pairs.

[h] Significantly different, p = 0.022, unpaired *t* test.

[i] Significantly different, p = 0.006, paired *t* test, 20 common pairs.

[j] Significantly different, p = 0.035, unpaired *t* test.

[k] Significantly different, p < 0.0001, paired *t* test, 22 common pairs.

[l] Significantly different, p = 0.0004, paired *t* test, 29 common pairs.

who underwent high-dose chemotherapy followed by stem cell rescue were treated with the Theratope STn-KLH cancer vaccine. The rationale for this study was that immunotherapy is likely to be most effective against small tumor burdens and, because these patients would be expected to have low tumor burdens after high-dose chemotherapy, they would not produce high levels of immunosuppressive mucins (18,19).

Immunological reconstitution after high-dose chemotherapy and stem cell transplantation in cancer patients has been extensively studied. Responses to protein recall antigens (e.g., poliovirus or tetanus toxoid) tend to recover faster than responses to protein neoantigens (e.g., KLH) (32). Of particular interest for the present study is the observation that antibody responses to *Haemophilus influenzae* capsular polysaccharide conjugated to a protein carrier are higher than the responses to the polysaccharide alone (32,38). In the present study, the transplant patients had a median anti-OSM IgG titer of 1:160, which compares favorably with a median titer of 1:40 in cohorts of patients with metastatic ovarian and breast cancer vaccinated with Theratope STn-KLH cancer vaccine but not receiving high-dose chemotherapy and stem cell transplants (8,9). One important difference between the patients in the two studies is that only one patient in the present study had elevated serum CA27.29 (MUC1 mucin) on trial entry compared with about one-half of the metastatic cancer patients in the nontransplant study. This observation is consistent with the hypothesis that serum MUC1 mucin is immunosuppressive (18,19) and that patients with elevated serum MUC1 levels generate a lower immune response to STn after immunization with Theratope STn-KLH cancer vaccine.

TABLE 6. *Daudi and OVCAR lysis by patient peripheral blood mononuclear cells not treated in vitro with interferon-2*

	First test		Peak post ASI[b]	
Pt. no.[a]	Daudi	OVCAR	Daudi	OVCAR
7	4.2	2.6	16.4 (4)	70.1 (4)
9	1.8	5.3	6.1 (4)	57.2 (4)
18	NT[c]	0.7	2.1 (1)	24.0 (2)
20	1.7	8.2	5.5 (4)	22.6 (1)
26	0	3.6	0 (3)	35.2 (3)
36	1.1	0	NT	23.3 (3)

[a] Patients whose peak OVCAR lysis was >22% ^{51}Cr release.

[b] Peak % specific ^{51}Cr release after immunization (no.).

[c] Not tested.

Blood CD4$^+$ T-cell levels are very low during the first 1–3 months after stem cell grafting and gradually rise to still subnormal levels during the next 5 years (32). As expected, we also observed very low CD4$^+$ counts in the present study (data not shown). Interestingly, the rising CD4$^+$ T-cells are largely composed of memory CD4$^+$ T cells, and the recovery of naive T-cell counts is extremely slow (32). The CD4$^+$ T-cell responses to polyclonal stimuli are subnormal, reflecting the very low CD4$^+$ T-cell counts. Few studies have evaluated the functional status of antigen-specific CD4$^+$ T-cells after grafting (32). The present study demonstrates potent T-cell proliferative responses to KLH and weak-to-strong T-cell responses to STn after immunization of transplant patients with Theratope STn-KLH cancer vaccine. The

TABLE 7. *"Interleukin (IL)-2-assisted" OVCAR specific killing*

Pt. no.	Peak OVCAR lysis[a]	OVCAR-Daudi peak lysis[b]	Pt. no.	Peak OVCAR lysis	OVCAR-Daudi peak lysis[b]
1	47.0 (3)	3.1	18	10.2 (1)	3.4
2	50.3 (2)	2.3	19	38.0 (4)	22.1
3	53.2 (2)	−2.5	20	36.9 (4)	13.3
4	28.2 (2)	3.3	21	14.6 (3)	0.8
5	14.3 (3)[c]	6.0	22	62.1 (3)	15.9
6	45.2 (1)	4.6	23	44.3 (2)	2.7
7	85.2 (3)[c]	53.4	24	55.1 (2)	13.3
9	50.6 (2)	−4.9	26	34.9 (3)[c]	10.8
11	61.8 (1)	15.2	27	91.7 (1)	19.5
12	50.8 (2)[c]	−1.9	30	67.5 (1)	11.5
13	8.6 (3)	7.3	31	63.0 (2)[c]	−22.6
14	54.6 (4)[c]	31.4	32	92.3 (1)[c]	52.5
15	97.8 (3)[c]	58.6	33	27.2 (1)	14.1
16	22.1 (1)	−0.6	34	23.1 (1)	7.2
17	45.6 (1)[c]	20.5			

[a] Peak specific percent ^{51}Cr release from OVCAR cells (10:1 E:T) [after (no.) immunizations] for patients who were tested for both OVCAR and Daudi "IL-2-assisted" lysis.

[b] Peak specific percent ^{51}Cr release of Daudi kill subtracted from peak specific percent ^{51}Cr release of OVCAR kill.

[c] Peak specific lysis of OVCAR and Daudi occurred on different days, otherwise occurred on the same day.

strength of the T-cell response to KLH in our patients might reflect an efficient immunization protocol in stem cell transplant patients because skin testing showed subnormal responses to KLH for 2–3 years after bone marrow grafting (32,39).

Circulating $CD8^+$ T-cell levels are also subnormal during the first 3 months after stem cell grafting, after which they rise quickly and reach normal levels by 1 year posttransplant. Again, the recovered $CD8^+$ cells are largely composed of memory cells (32). In the present study, we observed the development of enhanced lytic capacity of the patients' PBL when tested with OVCAR cells. Further studies will be required to determine whether this enhanced lytic capacity after immunotherapy is due to reactivation of memory-type $CD8^+$ CTL. Finally, we observed decreased NK function in our transplant patients compared with normals, which recovered after immunotherapy. This observation is consistent with previous reports that NK function becomes normal as early as 1 month posttransplant (40). Therefore, the increase in NK and LAK lysis postimmunotherapy may simply be due to the recovery of the immune system post high-dose chemotherapy and stem cell transplant and not due to the immunization.

Although antibody titers were determined using solid-phase synthetic STn in ELISA, it was considered that the IgG response to STn measured using solid-phase OSM was the most clinically relevant in these studies, because previous studies showed a correlation between the antibody titer to STn expressed on OSM and survival (8). The natural mucin OSM detects anti-STn IgG that binds preferentially to clustered STn rather than STn monomer (11) and the anticluster IgGs appear to be more clinically relevant (8,41). It is noteworthy in this regard that 16 of 30 evaluable patients attained an anti-STn OSM titer of 1:160 or greater, and many patients showed a sustained increase in titers to a high plateau titer. Although there was a direct correlation between the anti-STn titers and anti-OSM titers, there was no significant correlation of the anti-KLH IgG response with anti-STn OSM titers, which is consistent with our previous studies (8,9), suggesting that patients who generate a high immune response to STn after immunization with Theratope STn-KLH cancer vaccine are not simply a separate biologic subgroup of patients who would nonspecifically generate a high immune response to any antigen.

Evidence of the development of a TH_1 immune response to the STn epitope in these patients was obtained using a T-cell proliferation assay as well as antigen-specific cytokine production. In comparison to normal donors or the patients' preimmunotherapy T-cell proliferation, 17 of 27 evaluable patients who received at least three immunizations had a significantly higher SI in response to STn. Eleven of the patients showed evidence of STn antigen-specific production of IFN-γ but no significant IL-4 production, consistent with the hypothesis that a TH_1 response to STn is being measured in these cultures.

In this study, the patients' PBLs, preincubated either in the presence or absence of IL-2 before the lytic assays,

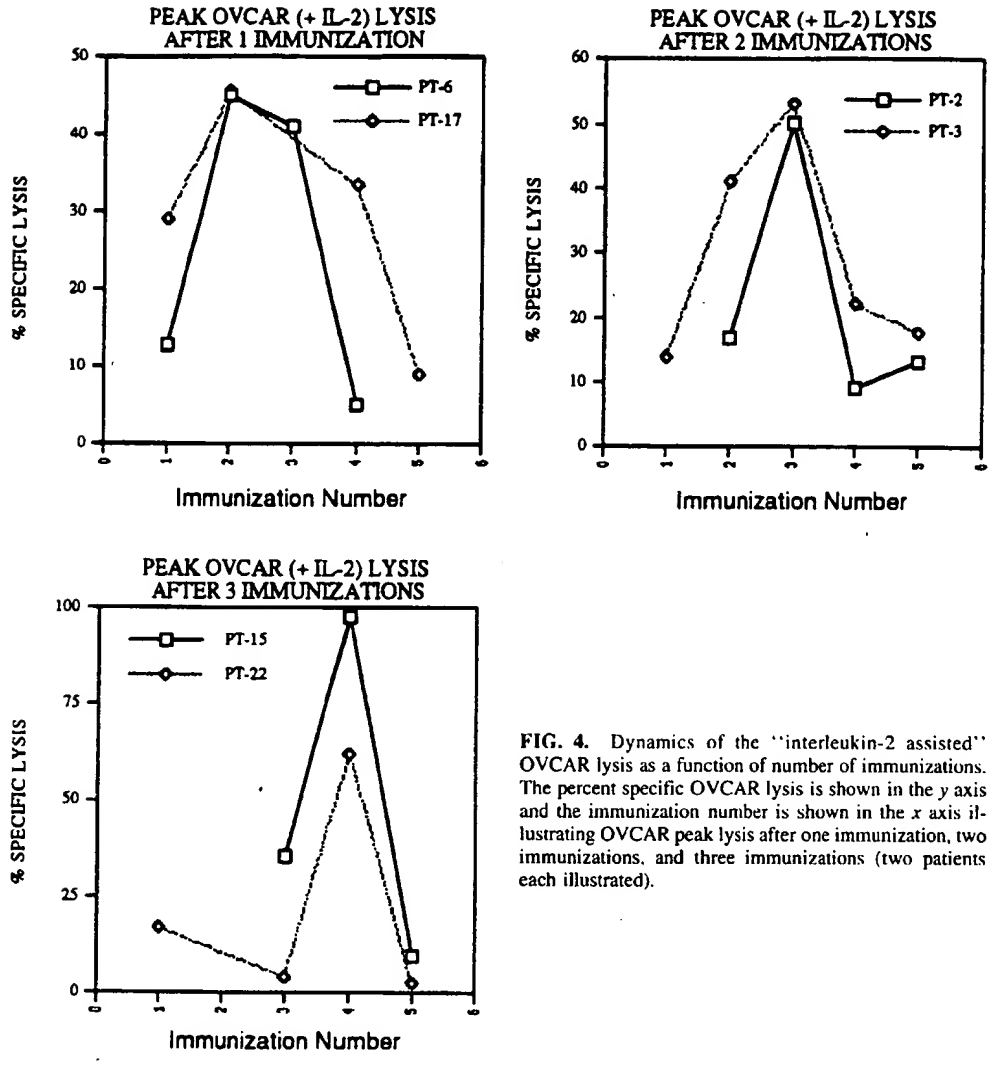

FIG. 4. Dynamics of the "interleukin-2 assisted" OVCAR lysis as a function of number of immunizations. The percent specific OVCAR lysis is shown in the *y* axis and the immunization number is shown in the *x* axis illustrating OVCAR peak lysis after one immunization, two immunizations, and three immunizations (two patients each illustrated).

were tested in parallel for lytic activity against K562, Daudi, and OVCAR STn^+ cancer-derived target cell lines. We found that NK lysis and OVCAR lytic activity of the patients tested preimmunotherapy or just after the first immunotherapy treatment was significantly lower than normal controls, whereas LAK lytic activity was not significantly different from that of the normals. After immunotherapy, NK, LAK, and OVCAR lytic activity increased significantly. In contrast, there is no significant increase in killing of Daudi cells (–IL-2) after immunotherapy, which is consistent with the hypothesis that there was an increase in specific killing of the STn^+ OVCAR cancer cell line after immunotherapy. This was further supported by the finding that six of the patients developed especially potent killing of the STn^+ OVCAR cells after immunotherapy, which was far greater than the maximum lysis of Daudi cells by the same patient. An increase in killing of the OVCAR target cells was noted after incubation of the patients' PBLs with IL-2 ("IL-2-assisted OVCAR killing"). IL-2-assisted OVCAR killing after immunotherapy was greater than the increase in LAK activity ("IL-2-assisted Daudi killing"). Using this criterion, 14 of the 29 evaluable patients had an increase in IL-2-assisted OVCAR lysis after immunotherapy. No definite conclusions with regard to antigen specificity of any punitive CTL killing of the STn expressing OVCAR target cells can be made at this time, although it is noteworthy that Daudi cells do not express STn. Further studies using multiple tumor target cell lines that express or do not express STn will be required to further define the specificity and possible major histocompatibility complex (MHC) restriction of

the enhanced cytotoxicity after immunotherapy with Theratope STn-KLH cancer vaccine. Nevertheless, the increase in lytic activity against the STn^+ cancer target after immunotherapy is encouraging. It is noteworthy in this regard that five of the seven patients who had pre-immunotherapy elevated CA125 levels showed decreasing levels during their immunotherapy, which is consistent with a therapeutic response (42); three of the seven patients (patients 1, 20, and 32) showed a particularly striking decrease in CA125 levels to the "normal" (<37 U/ml serum) range during immunotherapy. One patient (patient 36) demonstrated a dramatic drop in CA125 values, although not into the "normal" range, whereas one additional patient (patient 16) showed only a modest decrease.

Tn, T, and sialyl-Tn are O-linked, mucin-type, tumor-associated carbohydrate antigens that are highly expressed in human adenocarcinomas (31). Despite the promising clinical results suggesting that antibodies against "T-like" antigens are associated with a better clinical outcome (8,9,43,44), the issue of T-cell responses to tumor-associated carbohydrate antigens has received less attention. Mice immunized with Tn or T antigen coupled to KLH show prolonged survival after challenge with the highly lethal Tn and T-expressing tumor TA3-HA (23). Increased survival in T or Tn immunized mice correlated with the delayed-type hypersensitivity (29,44) or T-cell proliferation (30) to T-like antigens. Recent evidence has shown that murine T cells can specifically recognize carbohydrate epitopes (25–30). Most relevant for the present study is a recent report by Galli-Stampino and colleagues (30), who demonstrate specific T-cell recognition of Tn or T epitopes in CBA/J mice. In their studies, Tn or T epitopes stimulated strong, MHC class II restricted T-cell proliferation responses. In the present study, we provide evidence of T-cell proliferation and IFN-γ production in response to STn epitopes in humans. Further studies will examine the fine specificity of the T-cell response (e.g., in the present study we did not look for possible cross reactions to Tn, T, etc.). Nevertheless, we believe that this report provides the strongest evidence of a T-cell response to a defined carbohydrate epitope in humans.

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